Cloning and Characterization of the 2-C-Methyl-D-erythritol 4-Phosphate (MEP) Pathway Genes of a Natural-Rubber Producing Plant, Hevea brasiliensis

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Received June 10, 2008; Accepted July 15, 2008; Online Publication, November 7, 2008

[doi:10.1271/bbb.80387]

Natural rubber is synthesized as rubber particles in the latex, the fluid cytoplasm of laticifers, of Hevea brasiliensis. Although it has been found that natural rubber is biosynthesized through the mevalonate pathway, the involvement of an alternative 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is uncertain. We obtained all series of the MEP pathway candidate genes by analyzing expressed sequence tag (EST) information and degenerate PCR in H. brasiliensis. Complementation experiments with Escherichia coli mutants were performed to confirm the functions of the MEP pathway gene products of H. brasiliensis together with those of Arabidopsis thaliana, and it was found that 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase of H. brasiliensis were functionally active in the E. coli mutants. Gene expression analysis revealed that the expression level of the HbDXS2 gene in latex was relatively high as compared to those of other MEP pathway genes. However, a feeding experiment with [1-13C] 1-deoxy-D-xylulose triacetate, an intermediate derivative of the MEP pathway, indicated that the MEP pathway is not involved in rubber biosynthesis, but is involved in carotenoids biosynthesis in H. brasiliensis.

Key words: Hevea brasiliensis; natural rubber; cis-polyisoprene; rubber biosynthesis; 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway

Isoprenoids, widely found in nature, are a large group of compounds consisting of five-carbon isoprene units,
isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP). They have various biochemical functions. They are synthesized via the well-known mevalonate pathway and the recently discovered 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MEP pathway consists of seven enzymes (Fig. 1). The initial step in this pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by condensation of pyruvate and D-glyceraldehyde 3-phosphate (D-GAP), catalyzed by DXP synthase (DXS) in a thiamine diphosphate-dependent manner. This enzyme is also essential to the synthesis of vitamins B1 and B6 in bacteria. In the second step, intramolecular rearrangement and reduction of DXP simultaneously occur to form MEP. This reaction is catalyzed by NADP-dependent DXP reductoisomerase (DXR). MEP is finally converted into IPP and DMAPP by the action of the following enzymes: MEP cytidylyltransferase (CMS), 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME) kinase (CMK), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (cMEPP) synthase (MCS), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS), and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase (HDR). In higher plants, the mevalonate pathway and the MEP pathway function in cytoplasm and plastids respectively.

Hevea brasiliensis Müll. Arg. is a tree that produces natural rubber, an industrially invaluable isoprenoid polymer (cis-polyisoprene). Natural rubber is present as rubber particles in latex, which is the fluid cytoplasm of laticifers in H. brasiliensis. Natural rubber produced by H. brasiliensis has superior physical properties in comparison with synthetic rubber, and is an important raw material for rubber products such as truck, bus, and airplane tires and medical gloves.

In the 1960s, tracer experiments with [3-14C] 3-hydroxy-3-methyl-glutaryl (HMG)-CoA, an intermediate of the mevalonate pathway, revealed that isoprene units, the source of natural rubber, are synthesized via the mevalonate pathway. We recently isolated the mevalonate pathway genes by analyzing the EST information of latex and xylem cDNA libraries in H. brasiliensis, and observed high expression of the mevalonate pathway genes in latex as compared with other types of tissue.

On the other hand, Chow et al. conducted large-scale transcriptome analyses of the latex of H. brasiliensis and observed high expression of the DXS gene in the latex. Based on this observation, they suggested that the MEP pathway might be also involved in natural rubber biosynthesis, but the full extent of the MEP pathway of H. brasiliensis has yet to be discovered, and its involvement in rubber synthesis has not been confirmed.

In the present study, we obtained all the series of the MEP pathway candidate genes by analyzing EST information on H. brasiliensis together with degenerate PCR. The individual functions of these MEP pathway genes were evaluated by complementary assay using MEP pathway gene-disrupted E. coli mutants, and it was found that DXR, CMSs, and MCSs were functionally active in the E. coli mutants. In addition, the expression patterns of the MEP pathway genes in various tissues were also investigated in order to ascertain the physiological role of the MEP pathway genes. Feeding experiments with a 13C-labeled precursor were performed to determine MEP pathway involvement in rubber biosynthesis.
**Materials and Methods**

*Plant materials.* Various tissue samples were obtained from 10-year-old trees of *H. brasiliensis* (clone RRIM600) growing on commercial plantation PTPN VIII, Cikumpay, Purwakarta, West Java, Indonesia. Latex exuding from the tapped trees was collected and mixed with equal volumes of 2 × RNA extraction buffer (0.1 M Tris–HCl, 0.3 M LiCl, 0.01 M EDTA, and 10% SDS, pH 9.5), as described previously. Xylem tissues were obtained from young branches at an early stage of leaf development by peeling the phloem, including laticifers, and scraping both exposed surfaces with a scalpel. The collected samples were immediately frozen in liquid nitrogen and shipped back to the laboratory on dry ice.

**RNA isolation and cDNA synthesis.** Total RNA from the latex was isolated by a modification of the Cetyl trimethyl ammonium bromide (CTAB) method after the latex mixture was centrifuged at 10,000 × g for 20 min to remove the rubber component. Total RNA from other tissues was isolated using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan), and purified using an RNase-Free DNase Set (Qiagen) according to the manufacturer’s protocol. For 3’ RACE, the 3’-Full RACE Core set (Takara) was used with latex and xylem total RNA as the template. For real-time PCR, the WT-Ovation RNA Amplification System (Nugen Technologies, San Carlos, CA, USA) was used with 50 ng of each total RNA to synthesize single-strand cDNA.

**Isolation of cDNA encoding the MEP pathway genes.** For HbMCS, a partial fragment of the cDNA encoding HbMCS was obtained by degenerate PCR using one set of primers of YB2 and YB4, as described previously, since we failed to obtain partial sequence information for this gene from our EST database. To obtain the full-length sequences of the *H. brasiliensis* MEP pathway genes, 5’- and 3’-rapid amplification of cDNA ends (RACE) were conducted. For 5’ RACE, the 5’-Full RACE Core Set (Takara) was used with *H. brasiliensis* total RNA as the template and a set of specific primers, RT for reverse transcription, S1 and A1 for the first PCR, and S2 and A2 for the second PCR: HbDXS1_3’-S1 (5’-CATGTTCACCTGAGAAGAGGTCG-3’ and HbDXS1_3’-S2 (5’-AAGCTGAGATAAGTACCCAG-3’); HbDXS2_3’-S1 (5’-ACAGTGAAGTCTTGAGACCATATT-3’) and HbDXS2_3’-S2 (5’-GGACCTGTTCTCAGTTCAGTGT-3’); HbDXR_3’-S1 (5’-TCCTCCAC-CAGCGCTG-3’) and HbDXR_3’-S2 (5’-GGATCCACT-GGCTCC-3’); HbCMS1_3’-S1 (5’-GCAATGGCAAAGCTAATATC-3’) and HbCMS1_3’-S2 (5’-AGCAATAATGGTATATAAACCC-3’); HbCMK_3’-S1 (5’-GAAGAATTTTGGATATAGGACGATC-3’) and HbCMK_3’-S2 (5’-CTTATACAGGAAGAAGCAAGACT-3’); HbMCS1_3’-S1 (5’-TGCAATTTTGAGTATTAGGACG-3’) and HbMCS2_3’-S2 (5’-GCCCTGACATTTGGCCG-3’); HbHDS_3’-S1 (5’-CTATGACTACAGTGACTAAGTAGGA-TG-3’) and HbHDS_3’-S2 (5’-GACAATGTTAGGAATAGGACGAGACA-3’); and HbHDR_3’-S1 (5’-GAATTCTTGATTTGGGTGACC-3’) and HbHDR_3’-S2 (5’-GGCTAGCTACAGCCTTC-3’). All PCR products were cloned into pT7Blue T-vector (Takara) and sequenced. The sequence data were combined with partial EST sequences to obtain the full-length sequences of the *H. brasiliensis* MEP pathway genes.

**Construction and screening of leaf cDNA library.** A cDNA library was constructed in a PDONR222 vector according to the manufacturer’s protocol (Invitrogen, Tokyo, Japan) using mRNA prepared from leaves, as described above. A set of specific primers, HbMCS1_5’-S1 (5’-TTTACATAAATCAGATCGCTCG-3’ and HbMCS1_5’-S2 (5’-CATATGTTCTCCTGTAACAGTGA-3’); and HbMCS2_5’-S1 (5’-CAAAGGCTAGTCTGATCA-3’) and HbMCS2_5’-S2 (5’-AAATAGCATGATTTGGGGTCACC-3’); HbHDR_5’-S1 (5’-GAATTCTTGATTTGGGTGACC-3’) and HbHDR_5’-S2 (5’-GGCTAGCTACAGCCTTC-3’), were used to amplify partial fragments. Colony hybridization was performed, and then the cDNA clones hybridized to the probes were sequenced.

**Southern blot analysis.** Genomic DNA was isolated from leaves of *H. brasiliensis* (clone RRIM600) by a modification of the CBCT method. The genomic DNA was digested successively with BamHI, BglII, EcoRV, KpnI, PstI overnight, and then separated by electrophoresis in 0.8% agarose gels and blotted onto a nylon membrane (Hybond-N, GE Healthcare, Buckinghamshire, UK). The DNA probe was labeled with [32P]-dCTP using the Megaprime DNA Labelling System (GE Healthcare), and hybridization was performed at 65°C for 4 h. After hybridization, the blotted membrane was washed with primary wash buffer (1 × SSC, and 0.1% w/v SDS) at 65°C for 15 min, and with secondary wash buffer (0.2 × SSC, and 0.1% w/v SDS) at room temperature.
temperature. The blot was exposed on X-ray film (BioMax MS; Kodak, Corning, NY, USA) for 3 d at 4°C prior to development.

**Complementation assay.** To perform complementation assays of the MEP pathway genes, MEP pathway gene-disrupted *E. coli* mutants were used as follows: DXM3 for DXS, DYMI for DXR, NMW33 (pTVM20Km) for CMS, NMW29 (pTVM20Km) for CMK, NMW25 (pTVM20Km) for MCS, NMW18 (pTVM20Km) for HDS, and DLYT1 (pMMV225) for HDR. pTVM20Km is a pTVM20 derivative with 1.4-kb kan cassette (a kanamycin-resistant gene, aphII) from pKC7 reviewed into the Scal site in the amp' gene of pTVM20. The MEP pathway genes of *E. coli*, *A. thaliana*, and *H. brasiliensis* were cloned into a vector, pTTQ18, for the HDR gene, and into another vector, pMW118, (Nippon Gene, Tokyo) for the other genes. *H. brasiliensis* and *A. thaliana*, fragments encoding both immature (full-length ORF) and mature (transit peptide truncated ORF) proteins were amplified with their corresponding primer sets, cS1 and cA for immature protein, which contained N-terminal transit peptides, and cS2 and cA for mature protein, which did not contain N-terminal transit peptides, as follows: HbDXS1_cS1 (5'-TAILGACTCTGACGCTTCTCCTGCACCC-3'), HbDXS1_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), HbDXS1_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), HbDXS2_cS1 (5'-TAILGACTCTGACGCTTCTCCTGCACCC-3'), HbDXS2_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), HbDXS2_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), HbDXS1_cS1 (5'-TAILGACTCTGACGCTTCTCCTGCACCC-3'), HbDXS1_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), HbDXS1_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3'), MCMC_cS1 (5'-AIIGAGTGTCATCTCCTGAAGACCC-3'), MCMC_cS2 (5'-CATGAGATTGTCATCTCCTGAAGACCC-3'), MCMC_cA (5'-CAGTCGTAGCCTGATATCTGCGACCTACCACTTCC-3').
Gene expression analysis by real-time PCR. For quantification of *Hevea* MEP pathway genes transcripts in the various tissues, quantitative real-time PCR was performed with primers designed using Primer Express (Applied Biosystems, CA, USA), as follows: HbDXS1-qFw (5'-CAGAGCAGAAGAAGATGTGCAAGGAT-3') and HbDXS1-qRv (5'-AACAGCAAGAAGAGAAGATTTAG-3'); HbDXS2-qFw (5'-TGGTTGTTGTGTTGCA-GATG-3') and HbDXS2-qRv (5'-CTACCATGTGCGACAAGCAGCAA-3'); HbHDR-qFw (5'-ACCCCTCTATGGATCCTGGCCTATG-3') and HbHDR-qRv (5'-GCGACTAAGCCTCCAGATCAG-3'); HbCMS1-qFw (5'-CGCTGCCAGAAGAAGAGATTTG-3') and HbCMS1-qRv (5'-GCTTGACACCATCTTGGTGAC-3'); HbHDS-qFw (5'-AGGTTCTATGGCAAGAAGATGTG-3') and HbHDS-qRv (5'-GGCAAGACCCGACCTCAG-3') and HbHDS-qFw (5'-AACCTGGATGCCCACC-TTGATTC-3') and HbCMS2-qRv (5'-AGCTGACACATCCAGATCAG-3').

Table 1. *E. coli* Mutant Information

<table>
<thead>
<tr>
<th>Disrupted gene</th>
<th>Mutant name</th>
<th>Requirement for growth</th>
<th>Resistant gene</th>
<th>References</th>
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<tr>
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<td>kanamycin</td>
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</tr>
<tr>
<td>DXR</td>
<td>DYM1</td>
<td>ME</td>
<td>kanamycin</td>
<td>13</td>
</tr>
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<td>MVA</td>
<td>kanamycin</td>
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<td>HDR</td>
<td>DLYT1^2</td>
<td>MVA</td>
<td>kanamycin</td>
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</table>

^1harboring pTMV20KM plasmid; ^2harboring pMMV22S plasmid

‘Feeding experiment. Four ml of 0.25% [13C]-isotopomer solution containing 0.02% Tween 80 was aseptically added to the test tubes. The test tubes were incubated in a growth chamber at 25°C (16 h light/8 h dark) for 30 d. The test tubes were shaken once every 2 d, and the whole plant was drenched with the [13C]-isotopomer solution.

Sample preparation. To extract rubber (cis-polyisoprene), latex was collected into ethanol from *H. brasiliensis* seedlings (35-day old) were used for the feeding experiment.

LC-MS analysis of β-carotene. The mass spectrum of β-carotene was acquired by direct infusion analysis using ACQUITY UPLC (Waters, Milford, MA, USA) coupled with time-of-flight mass spectrometry (LCT...


Table 2. *H. brasiliensis* EST Clones Annotated as MEP Pathway Genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Tree no.</th>
<th>Number of EST clones</th>
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<td>1-Deoxy-d-xylulose-5-phosphate synthase (DXS)</td>
<td>05597</td>
<td>2 (0:2)</td>
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<tr>
<td>1-Deoxy-d-xylulose-5-phosphate reductoisomerase (DXR)</td>
<td>01902</td>
<td>1 (1:0)</td>
</tr>
<tr>
<td>2-C-methyl-d-erythritol 4-phosphate cytidylyltransferase (CMC)</td>
<td>00930</td>
<td>1 (1:0)</td>
</tr>
<tr>
<td>4-(Cytidine 5'-diphospho)-2-C-methyl-d-erythritol kinase (CMK)</td>
<td>01777</td>
<td>1 (0:1)</td>
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<tr>
<td>2-C-methyl-d-erythritol 2,4-cyclophosphosphate synthase (MCS)</td>
<td>10832</td>
<td>1 (1:0)</td>
</tr>
<tr>
<td>4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS)</td>
<td>—</td>
<td>N.D.</td>
</tr>
<tr>
<td>4-Hydroxy-3-methylbut-2-en-1-yl diphosphate reductase (HDR)</td>
<td>09077</td>
<td>1 (0:1)</td>
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<tr>
<td>—</td>
<td>11262</td>
<td>1 (0:1)</td>
</tr>
<tr>
<td>—</td>
<td>00233</td>
<td>3 (1:2)</td>
</tr>
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</table>

Data are from our two different cDNA libraries, latex and xylem. Tree no. represents arbitrary allocated numbers, when clustering analysis were performed based on sequence homology using VISUAL-BIO Clustering software (NTT SOFT, Tokyo). The numbers in brackets refer to the numbers of ESTs sequenced in the libraries.

Results and Discussion

Identification of the MEP pathway genes in *H. brasiliensis* by mining of EST database

Candidate ESTs for the MEP pathway in *H. brasiliensis* were identified by mining an in-house EST database representing cDNA libraries from *H. brasiliensis* RRIM600 latex and xylem. Partial sequences of DXS, DXR, CMS, CMK, HDS, and HDR were found (Table 2). In addition, two partial sequences of DXS and CMS were found, but no candidate ESTs of MCS were found in our EST database.

Isolation of the MEP pathway genes in *H. brasiliensis*

To obtain the full-length cDNA encoding putative DXS, DXR, CMS, CMK, HDS, and HDR in *H. brasiliensis*, the 5' and 3' RACE methods were applied using the various sequence-specific primers. Based on these sequences, full-length cDNAs were amplified and cloned in pT7Blue vector, and then sequenced.

To obtain information on MCS in *H. brasiliensis*, degenerate PCR was performed using primer sets described previously.

To obtain information on MCS in *H. brasiliensis*, degenerate PCR was performed using primer sets described previously. The deduced amino acid sequence encoded by 271 bp of the obtained PCR fragment showed high sequence similarity to MCS from *A. thaliana* and *Catharanthus roseus*. Subsequently, the 3'RACE and 5'RACE method were applied using sequence-specific primers. Two 3'RACE products of MCS were obtained. Unfortunately, we failed to obtain the 5' upstream region of MCS. Hence, cDNA library screening was performed using the two 3'RACE products as probes. Screening of the total of 8.0 × 10^4 colonies resulted in the identification of only two colonies hybridized to the each probe. The plasmids isolated from these colonies contained a full-length cDNA.

Finally, all of the MEP pathway genes in *H. brasiliensis* were obtained. The ORFs of *H. brasiliensis* were tentatively designated HbDXS1, HbDXS2, HbDXR, HbCMS1, HbCMS2, HbCMK, HbMCS1, HbMCS2, HbHDS, and HbHDR. The lengths of the cDNAs, and the open reading frames (ORFs), amino acid residues, theoretical pI, theoretical M_r, and GenBank accession numbers are shown in Table 3. At the DNA level, HbDXS1 showed 64.5% identity with HbDXS2, HbCMS1 showed 92.5% identity with HbCMS2. HbMCS1 showed 88.7% identity with HbMCS2.

Previous reports indicate that other plants possess two kinds of DXS genes, but a single copy of each CMS and MCS gene. To confirm the copy number of CMS and MCS in *H. brasiliensis*, Southern blot analyses were performed using the ORF sequences as a probes. Our results suggest that the copy number of each HbCMS and HbMCS is at least two (data not shown).
The MEP pathway genes of *H. brasiliensis* were aligned with those of *A. thaliana*, *O. sativa*, *M. truncatula*, and *E. coli* (Fig. 2). These results suggest that all of the MEP pathway genes of *H. brasiliensis* also have N-terminal plastid transit peptides, as those of other plants do.29)

The essential amino acid residues for catalytic activities and substrate binding in the MEP pathway genes of *E. coli* were highly conserved in *H. brasiliensis*.30–36) In addition, insertion sequences, whose function remains unknown, are highly conserved in plant HDS and HDR.37) These insertion sequences were also highly conserved in the HDS and HDR of *H. brasiliensis*.

**Phylogenetic analysis**

Phylogenetic trees were constructed based on the MEP pathway genes retrieved from GenBank in order to observe the evolutionary relationships of the plants to *E. coli* (Fig. 3).

In MEP pathway genes, *H. brasiliensis* genes are located in the plant gene clusters that are clearly distinct from *E. coli* ones. Among DXS genes, plant genes are mainly classified into two groups, DXS1 and DXS2, as reported previously.23–27) HbDXS1 and HbDXS2 were located in the DXS1 and the DXS2 group respectively. Except for *A. thaliana* DXS genes, molecular evolution from microorganisms to dicotyledonous species of angiosperm via gymnosperm and monocot nuer species was clearly observed in either DXS1 or DXS2, but in other MEP pathway genes of *H. brasiliensis*, it is difficult to observe any evolutionary relationship, because sequence information might still be limited.

**Functional identification of MEP pathway genes in *H. brasiliensis***

To confirm the catalytic functions of the cloned *H. brasiliensis* MEP pathway genes, we used the MEP pathway gene-disrupted *E. coli* mutants listed in Table 1. The mutants require supplemented ME or MVA for growth because disruption of the MEP pathway genes is lethal for *E. coli*. We expected that the individual MEP pathway genes cloned from *H. brasiliensis* would complement the defect of the mutant in each MEP pathway gene.

Unexpectedly, the DXM3 transformants harboring AtDXSs and HbDXS1 did not grow in the absence of supplemented ME (Fig. 4A), and the DXM3 transformant harboring HbDXS2 did not grow under the same conditions (data not shown), although it has been reported that DXS from *G. biloba* and *O. sativa* complemented the same gene-disrupted *E. coli* mutant DXM3.12,27) These results suggest that the expression level of the DXS genes of *A. thaliana* and *H. brasiliensis* are insufficient to complement the DXM3 mutant. Alternatively, DXS from *A. thaliana* and *H. brasiliensis* might be unable to maintain a proper protein fold in *E. coli*.

The DYM1 transformant harboring AtDXR regardless of presence of N-terminal transit peptides grew without additional ME (Fig. 4B). Also, the DYM1 transformant harboring HbDXR without N-terminal transit peptides grew without additional ME (Fig. 4B). These results indicate that HbDXR and AtDXR are also functionally active in *E. coli*.

The NMW3 transformant harboring HbCMSs or AtCMS regardless of presence of N-terminal transit peptides grew without additional MVA (Fig. 4C). These results indicate that HbCMSs and AtCMS are functionally active in *E. coli*. However, *E. coli* transformants with a plasmid containing the plant CMS gene with N-terminal transit peptides showed an abnormal watery phenotype, implying that plant transit peptides can lead to incorrect protein sorting in *E. coli*.

The NMW29 transformant harboring AtCMK regardless of presence of N-terminal transit peptides grew without additional MVA (Fig. 4D). These results indicate that AtCMKs are also functionally active in *E. coli*. However, the NMW29 transformant harboring

<table>
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<th>Tree no.</th>
<th>Enzyme</th>
<th>Full length (ORF)</th>
<th>Amino acid residue pI</th>
<th>Mₚ (kDa)</th>
<th>Accession no.</th>
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<td>05597</td>
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<tr>
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<td>HbDXS2</td>
<td>2439 (96–2231)</td>
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<td>76.3</td>
<td>AB294699</td>
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<tr>
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<td>311 7.69</td>
<td>34.2</td>
<td>AB294702</td>
</tr>
<tr>
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<td>462 5.62</td>
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<td>AB294708</td>
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Fig. 2. Continued.
HbCMK did not grow without additional MVA, although the essential residues for both catalytic activities and substrate binding are well conserved in HbCMK.

The NMW25 transformant harboring HbMCSs, or AtMCS, regardless of presence of N-terminal transit peptides, grew without additional MVA (Fig. 4E). These results indicate that HbMCSs and AtMCS are function-
Fig. 3. Phylogenetic Relationships of the MEP Pathway Genes.

The dendrogram was created using the ClustalW program. The genes in *H. brasiliensis* are boxed. Key and GenBank accession numbers as follows: A, DXS, HbDXS1 and HbDXS2 (this study); ApDXS (*Andrographis paniculata*, AAP14353); AtDXS1, AtDXS2, and AtDXS3 (*Arabidopsis thaliana*, ATU27099, and NM 121176); EcDXS (*Escherichia coli*, NP_414954); GbDXS1 and GbDXS2 (*Ginkgo biloba*, AAS89341 and AY494185); LeDXS (*Lycopersicon esculentum*, AAD38941); MdDXS1 and MdDXS2 (*Medicago truncatula*, CAD22530 and AJ30048); NpDXS (*Narcissus pseudonarcissus*, CAC08458); OsDXS1 and OsDXS2 (*Oryza sativa*, ABP81354 and ABP81355). B, DXR, HbDXR (this study); AtDXR (*A. thaliana*, NP_201085); AmDXR (*Antirrhinum majus*, AAW28998); ArDXR (*Artemisia annua*, AAD56391); CaDXR (*Camptotheca acuminate*, ABC86579); CcDXR (*Cistus incanus subsp. creticus*, AAP56260); EcDXR (*E. coli*, NP_414715); GbDXR (*G. biloba*, AAR95700); HvDXR (*Hordeum vulgare subsp. vulgare*, CAE47438); LuDXR (*Linum usitatissimum*, CAF22092); MdDXR (*Mentha piperita*, Q9XES0); PeDXR (*Picea abies*, ABS50518); TeDXR (*Tagetes erecta*, AAG10432); VvDXR (*Vitis vinifera*, CAO71043); ZmDXS1 and ZmDXS2 (*Zea mays*, ABP81354 and ABP81355). C, CMS, HbCMS1 and HbCMS2 (this study); AtCMS (*A. thaliana*, AAF61714); EcCMS (*E. coli*, NP_417227); GbCMS (*G. biloba*, AAZ80386); OsCMS (*O. sativa*, BAD29384); SrCMS (*S. rebaudiana*, ABB88836). D, CMK, HbCMK (this study); AtCMK (*A. thaliana*, NP_180261); EcCMK (*E. coli*, NP_415726); GbCMK (*G. biloba*, AAZ80386); LeCMK (*L. esculentum*, AAO15447); CrCMK (this study); MmCMK (*Medicago truncatula*, CAD22530); MsCMK (*Medicago sativa*, AAP56260); MsCMK (*M. sativa*, CAD22530); SoCMK (*Solanum tuberosum*, AAM62786); VvCMK (*V. vinifera*, CAO47671). E, MCS, HbMCS1 and HbMCS2 (this study); AtMCS (*A. thaliana*, AAM62786); BrMCS (*Brassica rapa*, BAF81514); CjMCS (*Citrus javanica*, BAF73931); EcMCS (*E. coli*, NP_417227); GbMCS (*G. biloba*, AAZ80386); GbMCS (*G. biloba*, AAZ80386); SrMCS (*S. rebaudiana*, ABB88836); TmMCS (*Taxus media*, ABB88836). F, HDS, HbHDS (this study); AtHDS (*A. thaliana*, AA01544); ChDS (*Cuscuta elegans*, AA02477); EcHDS (*E. coli*, NP_417201); GbHDS (*G. biloba*, ABP88087); LeHDS (*L. esculentum*, AAO15447); OsHDS (*O. sativa*, BAD19354); ShHDS (*S. rebaudiana*, ABG23395); TmHDS (*T. media*, ABB88836); VhHDS (*V. vinifera*, CAN72185). G, HDR, HbHDR (this study); AaHDR (*Adonis pulchella*, AA02477); AaHDR (*A. thaliana*, AA01544); AtHDR (*A. thaliana*, AA01544); ChHDR (*Cuscuta elegans*, AA02477); EcHDR (*E. coli*, NP_417201); GbHDR (*G. biloba*, ABP88087); LeHDR (*L. esculentum*, AAO15447); OsHDR (*O. sativa*, BAD19354); ShHDR (*S. rebaudiana*, ABG23395); VhHDR (*V. vinifera*, CAO47671).
The reducing shuttle system, though it is utilized in plants, cannot use NADPH/flavodoxin/flavodoxin reductase as a catalytic system due to their high sequence similarity to those of other plants.

In conclusion, we confirmed that HbDXS2, HbCMSs, and HbMCSs were functionally active in the corresponding E. coli mutants. On the other hand, the functions of HbDXSs, HbCMK, HbHDS, and HbHDR could not be confirmed by complementation experiments, but HbDXS, HbCMK, HbHDS, and HbHDR gene products functioned, as MEP pathway enzymes in H. brasiliensis due to their high sequence similarity to those of other plants.

**Gene expression pattern of MEP pathway genes**

To observe the expression patterns of MEP pathway genes in different tissues of H. brasiliensis, total RNA was isolated from red young leaf, pale green young leaf, mature leaf, latex, phloem, and xylem and used in quantitative real-time PCR. In H. brasiliensis, leaf tissues drastically changed their morphology and metabolites, such as chlorophyll, during development (Fig. 5A–C). The gene expression levels of all MEP pathway genes except for HbDXS2 increased with leaf development and were highest in the mature leaf, but extremely low in latex. The HbCMSs (HbCMS1 and HbCMS2) and the HbMCSs (HbMCS1 and HbMCS2) showed similar expression patterns. In contrast, the gene expression level of HbDXS2 was highest in latex.

Previous studies indicate that DXS1 is expressed in photosynthetic tissues and is involved in primary metabolism, whereas DXS2 is expressed in specialized tissues and is involved in specialized isoprenoid biosynthesis. For example, *Medicago truncatula* accumulates apocarotenoid in mycorrhizal roots.23) *Ginkgo biloba* synthesizes diterpene ginkgolide in roots.26) *Mentha piperita* synthesizes and accumulates monoterpenes in oil gland secretory cells.40) *Picea abies* synthesizes and accumulates oleoresin in the resin duct.25) Biosynthesis is induced by external stimuli such as wounding, fungal attack, and the methyl jasmonate treatment.18,25) Our results in the gene expression analysis of HbDXSs (HbDXS1 and HbDXS2) showed similar expression patterns. In contrast, the gene expression level of HbDXS2 was highest in latex.

**E. coli**38,39) These reports explain our result that HDS and HDR from *A. thaliana* and *H. brasiliensis* failed to complement the defect of the E. coli mutants. In contrast, HDR from *G. biloba* complemented the HDR gene-disrupted E. coli mutant DLYT1(pMMV22S).18) These results suggest that the reducing shuttle system for catalytic activity in HDR is different between gymnosperm and angiosperm.

**Fig. 4. Functional Complementation.**

A, 1, wild-type *E. coli* FS1576; 2, *E. coli* DXS knock-out mutant DXM3; 3, gdx-pTT;12,4 EcDXS; 5, immature AtDXS1; 6, mature AtDXS1; 7, immature AtDXS2; 8, mature AtDXS2; 9, immature AtDXS3; 10, mature AtDXS3; 11, immature HbDXS1; 12, mature HbDXS1. B, 1, wild-type *E. coli* FS1576; 2, *E. coli* DXR knock-out mutant DMY1; 3, pMEW41;13 EcDXR; 5, immature AtDXR; 6, mature AtDXR; 7, immature HbDXR; 8, mature HbDXR. C, 1, wild-type *E. coli* W3110; 2, *E. coli* CMS knock-out mutant NMW33; 3, pMNW33;14 EcCMS; 5, immature AtCMS; 6, mature AtCMS; 7, immature HbCMS1; 8, mature HbCMS1; 9, immature HbCMS2; 10, mature HbCMS2. D, 1, wild-type *E. coli* W3110; 2, *E. coli* CMK knock-out mutant NMW29; 3, pMNW29;15 EcCMK; 5, immature AtCMK; 6, mature AtCMK; 7, immature HbCMK; 8, mature HbCMK. E, 1, wild-type *E. coli* W3110; 2, *E. coli* HDS knock-out mutant NMW18; 3, pMNW18;17 EcHDS; 5, immature AtHDS; 6, mature AtHDS; 7, immature HbHDS; 8, mature HbHDS. F, 1, wild-type *E. coli* FS1576; 2, *E. coli* HDR knock-out mutant DLYT1; 3, pLYTB2.5;18 EcHDR; 5, immature AtHDR; 6, mature AtHDR; 7, immature HbHDR; 8, mature HbHDR.
whether natural rubber is synthesized through the MEP pathway or the MVA pathway in *H. brasiliensis*.

**Feeding experiment**

To investigate MEP pathway involvement in rubber biosynthesis in *H. brasiliensis*, feeding experiments in seedlings were performed with [1-13C] DX-3Ac and [2-13C] MVA, an intermediate of the MEP pathway, and with [13C]-labeled mevalonolactone (MVA), an intermediate of the MVA pathway as a control. Due to the hydrophilicity of DX, [1-13C] 1-deoxy-D-xylulose-3,4,5-triacetate ([1-13C] DX-3Ac) was used in this study to increase incorporation efficiency into the plant.

To confirm the incorporation of [1-13C] DX-3Ac and [2-13C] MVA into the plants, l/C12-carotene and l/C12-sitosterol were chosen as representative compounds via the MEP and MVA pathways respectively.

3) Isoprenoids from *H. brasiliensis* seedlings were extracted and analyzed. LC-MS analysis showed that l/C12-carotene was labeled in the case of feeding with [1-13C] DX-3Ac (Table 4), while GC-MS showed that l/C12-sitosterol was labeled in the case of feeding with [2-13C] MVA (Table 5). These results indicate that labeling took place.

Next, a soluble rubber fraction, extracted from seedling latex, was analyzed by 13C-NMR to identify the enriched position of the isoprene units. Only the C-5 position of the isoprene units should be enriched after [1-13C] DX-3Ac incorporation, while only the C-4 position should be enriched after [2-13C] MVA incorporation. Enrichment of C4 carbon was clearly observed in the case of feeding with [2-13C] MVA, as previously reported (Table 6, Fig. 6A). On the other hand, no enrichment of C4 carbon was observed in the case of feeding with [1-13C] DX-3Ac (Table 6, Fig. 6B).

Based on these results, we conclude that rubber is biosynthesized via the MVA pathway, but not via the MEP pathway. In addition, HbDXS2, expressed highly in laticifer, is most likely involved in carotenoid biosynthesis rather than in rubber biosynthesis in *H. brasiliensis*.
Fig. 6. $^{13}$C-NMR Spectra of cis-Polyisoprene from *Hevea brasiliensis* Fed with [2-$^{13}$C] MVA (A), and Fed with [1-$^{13}$C] DX-3Ac (B).

The intensities of the two signals at 135 ppm in the two $^{13}$C-NMR spectra were aligned. An arrow indicates an enriched signal as compared to that of the other positions.

![Chemical shift (ppm from TMS)](image)

Table 6. $^{13}$C-NMR Relative Peak Area

<table>
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<th>$^{13}$C-NMR relative peak area</th>
<th>C3</th>
<th>C2</th>
<th>C4</th>
<th>C1</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-$^{13}$C] DX-3Ac</td>
<td>100</td>
<td>193 ± 15</td>
<td>201 ± 12</td>
<td>204 ± 13</td>
<td>198 ± 22</td>
</tr>
<tr>
<td>[2-$^{13}$C] MVA</td>
<td>100</td>
<td>190 ± 8</td>
<td>354 ± 18</td>
<td>197 ± 8</td>
<td>173 ± 6</td>
</tr>
<tr>
<td>control</td>
<td>100</td>
<td>200 ± 9</td>
<td>205 ± 18</td>
<td>194 ± 14</td>
<td>181 ± 5</td>
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</table>

Peak areas of isoprene units were calculated relative to the C3 position. Results are given as average of three independent determinations ± standard deviation.

Acknowledgments

Part of this work was performed in the project “Development of Fundamental Technologies for Controlling the Production of Industrial Materials by Plants” supported by the New Energy and Industrial Technology Development Organization of Japan (NEDO). We are very grateful to Badan Pengkajian dan Penerapan Teknologi of Indonesia (BPPT) for provision of plant materials, and to Mr. E. Hirosue and Mr. S. Hayashi (Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Japan) for technical support.

References


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